Expression and engineering of hydrophobin genes

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Chapter 1: General introduction

General introduction

Filamentous fungi generally secrete small, moderately hydrophobic proteins first discovered in *Schizophyllum commune* and called hydrophobins (Wessels *et al*., 1991). They fulfil a broad spectrum of functions in fungal growth and development. Hydrophobins mediate escape of hyphae from a hydrophilic environment (Wösten *et al*., 1999) and are involved in formation of hydrophobic aerial structures like aerial hyphae (Wösten *et al.,* 1993; 1994b; van Wetter *et al.*, 1996; Bowden *et al*., 1996; Temple *et al*., 1997), spores (Beever and Dempsey, 1978; Stringer *et al*., 1991; Bell-Pedersen *et al*., 1992; Lauter *et al*., 1992; Talbot *et al*., 1996) and fruiting bodies (de Groot *et al*., 1996; Lugones *et al*., 1996). Moreover, they provide gas channels in fruiting bodies and in lichens with a hydrophobic lining, probably ensuring efficient gas exchange under wet conditions (Wessels *et al*., 1993; 1996; Lugones *et al*., 1999b; van Wetter *et al*., 2000; Scherrer *et al*., 2000). Hydrophobins also mediate attachment of hyphae to hydrophobic surfaces (Wösten *et al*., 1994a; van Wetter *et al*., 2000) and sensing thereof (Talbot *et al*., 1996), which is important in initial steps of pathogenic interactions before penetration and infection can occur. Recently, it was found that hydrophobins are also involved in cell wall assembly (van Wetter, 2000).

Hydrophobins show diverse amino acid sequences but they are all characterized by the presence of eight cysteine residues in conserved spacing and similar hydropathy patterns (Wessels, 1994; 1997; Wösten and Wessels, 1997). The most characteristic feature of hydrophobins is that they self-assemble into an amphipathic membrane when they are confronted with a hydrophilic-hydrophobic interface. This property makes them interesting candidates for use in medical and technical applications (Wessels, 1997). Bulk production and the possibility to engineer hydrophobins would enhance the likelihood of using hydrophobins in such applications. In this Introduction the properties of hydrophobins will be discussed and their role in fungal development will be illustrated by the functions of the SC3 hydrophobin. In addition, possible applications of hydrophobins will be described as well as the current state of (heterologous) protein production in filamentous fungi.

Hydrophobins

Biochemical and biophysical properties of hydrophobins

Hydrophobins are small (± 100 amino acids), moderately hydrophobic proteins, which are characterized by eight conserved cysteine residues (Figure 1A) and typical hydropathy patterns (Wessels, 1997). Based on mass-spectra and chemical analysis, it was found that hydrophobins may be post-translationally modified. For instance, the mature SC3 hydrophobin of *S. commune* contains 16-22 mannose residues (de Vocht *et al*., 1998, Chapter 2), which are probably attached to the 12 threonine residues in the long *N*-terminal stretch preceding the first cysteine residue (Figure 1B). In contrast, the mature forms of the SC4 hydrophobin of *S. commune* and ABH3 of *Agaricus bisporus* are not modified. These

hydrophobins do not contain a long *N*-terminal sequence preceding the first cysteine residue (Figure 1B) (Lugones *et al*., 1998; 1999b).

Based on differences in hydropathy patterns and biophysical properties, class I and class II hydrophobins are distinguished (see below, Wessels, 1994). The cysteine residues of the class I hydrophobin SC3 (de Vries *et al*., 1993) and the class II hydrophobin CU (Yaguchi *et al*., 1993) form intramolecular disulphide bridges. The cysteine linkages in CU were determined (Yaguchi *et al*., 1993) and assuming that disulphide bridges in class I hydrophobins are identical to those of CU, both hydrophobin classes seem to contain two similar domains (Figure 1B). Cysteine residues 1 to 4 are contained in the first domain, while the second domain encompasses cysteine residues 5 to 8. The presence of two domains is also indicated by their hydropathy patterns (Wessels, 1994; 1997) and by the similarity of the most prevalent amino acids surrounding the fourth and eight cysteine residues of class I hydrophobins (Wösten and Wessels, 1997). In the class I hydrophobins, the cysteine doublets (Cys2Cys3 and Cys6Cys7) are followed by a stretch of hydrophilic amino acids, whereas in class II hydrophobins, hydrophobic amino acids immediately follow the cysteine doublets. Also, fewer amino acids separate the third and fourth cysteine residue in class II hydrophobins when compared to class I hydrophobins (Figure 1A). Recently, a new type of hydrophobin was isolated from *Claviceps fusiformis* (de Vries *et al*., 1999). The mature hydrophobin, CFTH1, consists of three class II hydrophobin domains separated by glycin-asparagin rich regions. It shows biophysical properties similar to those of other class II hydrophobins.

Both class I and class II hydrophobins self-assemble at hydrophilic-hydrophobic interfaces (e.g. between water and air, water and oil, or water and a hydrophobic solid like Teflon) into an amphipathic membrane. This was shown for the class I hydrophobins SC3 (Wösten *et al*., 1993; 1994a; 1995), ABH1, ABH3 and SC4 (Lugones *et al*., 1996; 1998; 1999b), and the class II hydrophobins CU (Richards and Takai, 1973; Takai and Richards, 1978; Russo *et al*., 1982; Richards, 1993) and CRP (Carpenter *et al*., 1992). The membranes formed by class I hydrophobins are highly insoluble, that is, they resist hot SDS extraction and can only be dissociated by agents like formic acid and trifluoroacetic acid (TFA) (Wessels *et al*., 1991; de Vries *et al*., 1993). In contrast, assemblages formed by class II hydrophobins are less stable. Those of CU and CRP can be dissociated in 60% ethanol and 2% SDS (Russo *et al*., 1982; Carpenter *et al*., 1992; Wösten and de Vocht, 2000) and assembled CU also dissociates by applying pressure or by cooling (Russo *et al*., 1982).

The best characterized class I hydrophobin is SC3 of *S. commune* but other members of this class have similar properties. Upon contact with hydrophilic-hydrophobic interfaces, SC3 monomers self-assemble into a 10 nm thick amphipathic membrane (Wösten *et al*., 1993; 1994a; 1995). The hydrophilic side of the SC3 membrane has no clear ultrastructure and has a water contact angle (θ) of about 36°. On the other hand, the hydrophobic side is characterized by a mosaic of parallel rodlets and exhibits a water contact angle of 110°(which is as hydrophobic as Teflon)(Wösten *et al*., 1993; 1994a). Self-assembly at the water-air interface is accompanied by an increase in β-sheet structure (de Vocht *et al*., 1998) and by a

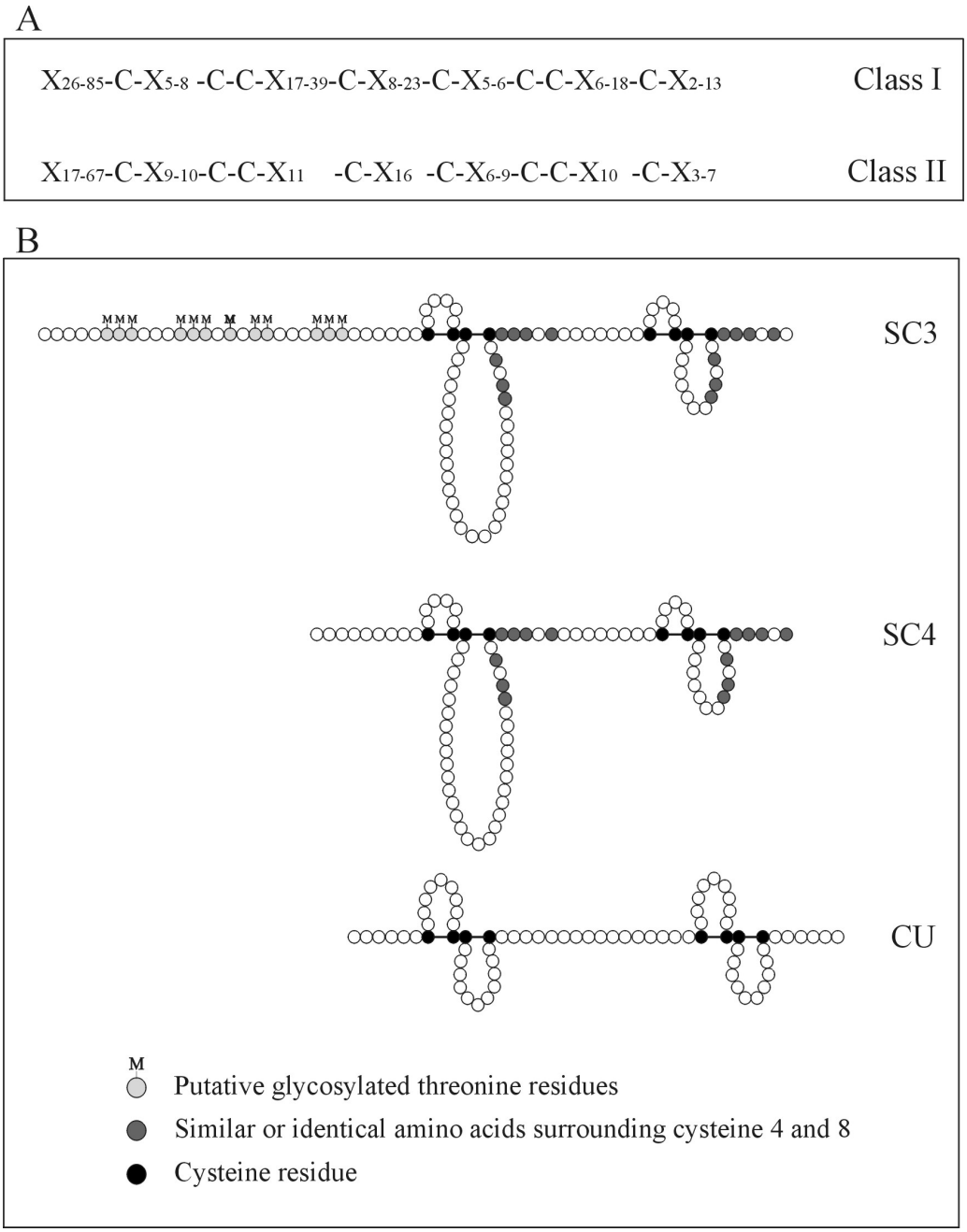

Figure 1. Primary structure of class I and class II hydrophobins. **(A)** The length of the sequences spacing the cysteine residues in class I and class II hydrophobins. The amino acid sequence preceding the first cysteine residue includes the signal sequence that is cleaved off during secretion. **(B)** Assuming cysteine linkages similar to those in the class II hydrophobin cerato-ulmin (CU), hydrophobins seem to be two-domain proteins. This is strengthened by the finding that in class I hydrophobins the fourth and the eighth cysteine residue are preceded by L-V/I-X-Pho and X (X indicates any amino acid except for W, Pho any hydrophobic amino acid) and are followed by S/T-P-I-X and V/I residues. Indicated are the class I hydrophobins SC3 and SC4 of *S. commune* and the class II hydrophobin CU of *Ophiostoma ulmi*.

reduction of the water surface tension from 72 mJ m^{-2} to as low as 24 mJ m^{-2} (van der Vegt *et al*., 1996; Wösten *et al*., 1999). The conformation of the monomers apparently changes in such a way, that hydrophilic and hydrophobic groups orient themselves at opposite sides of the membrane. Self-assembly at the water-Teflon interface is accompanied by an increase in α-helix structure (de Vocht *et al*., 1998). This conformation appears to be an intermediate of the β-sheet form found at the water-air interface. Upon treatment of SC3 with detergents like SDS and Tween-80 at 85 °C, SC3 at the Teflon surface attains the β-sheet form (Wösten and de Vocht, 2000; M. L. de Vocht, unpublished). Both the α-helix and β-sheet forms strongly adhere to hydrophobic surfaces and cannot be removed by washing with water but the α-helix form can be removed by cold detergents.

Little is known about the 3-D structure of hydrophobins. The mannose residues of SC3 appear to be exposed at the hydrophilic side of assembled SC3 as was indicated by X-ray photoelectron spectroscopy (Wösten *et al*., 1994c). The *N*-terminal part contains putative *O*-glycosylation sites (Figure 1B; de Vocht *et al*., 1998; Chapter 2) and in the absence of *N*-glycosylation sites in the SC3 molecule (Chapter 2) it is thus expected that the *N*-terminal part is glycosylated and exposed to the hydrophilic side. The same may apply to the POH2 hydrophobin of *Pleurotus ostreatus* (Ásgeirsdóttir *et al*., 1998). Most other hydrophobins, like ABH3 and SC4, do not contain a long *N*-terminal region preceding the first cysteine residue and do not contain putative *O*-glycosylation sites (Lugones *et al*., 1998; 1999b). However, both SC3 and SC4 assemble at hydrophobic-hydrophilic interfaces into very similar amphipathic membranes characterized by 10 nm wide rodlets.

The role of SC3 in fungal growth and development

The filamentous fungus *S. commune* is a homobasidiomycete that colonizes moist solid substrates like wood. Upon germination of spores, a monokaryotic mycelium is formed consisting of filaments that grow at their apices. The fungus initially grows submerged but after a feeding mycelium has been established sterile aerial hyphae are formed. Two monokaryons can form a fertile dikaryon when they contain different *MAT*A and *MAT*B mating type loci (for reviews see Kothe, 1996; Casselton and Olesnicky, 1998). This dikaryon not only forms aerial hyphae but also fruiting bodies in which spores are produced. *S. commune* contains at least four hydrophobin genes: *SC3*, *SC1*, *SC4* and *SC6,* all encoding class I hydrophobins (Schuren and Wessels, 1990; Wessels *et al*. 1995; de Vocht *et al.*, 1998; Lugones, 1998; Chapter 2). *SC1*, *SC4* and *SC6* are specifically expressed in the fruiting dikaryon, while *SC3* is expressed in both monokaryons and dikaryons. SC3 is involved in formation of aerial hyphae (Wösten *et al*., 1993; 1994b; 1999; van Wetter *et al.*, 1996), in attachment of hyphae to hydrophobic surfaces (Wösten *et al*., 1994a; van Wetter *et al*., 2000), and in cell wall assembly (van Wetter, 2000).

To grow into the air, hyphae are confronted with the high water surface tension (72 mJ m^{-2}) of the water film surrounding the hyphae. This surface tension appears to be a barrier for aerial growth (Wösten *et al*., 1999). When a sufficient amount of submerged mycelium is formed which can support growth of aerial hyphae and fruiting bodies, the *SC3* gene is

expressed. SC3 secreted by submerged hyphae diffuses to the water-air interface where it self-assembles into an amphipathic membrane (Figure 2A). This is accompanied by a huge drop in water surface tension (to as low as 27 mJ m^{-2}), enabling hyphae to grow into the air (Wösten *et al*., 1999).

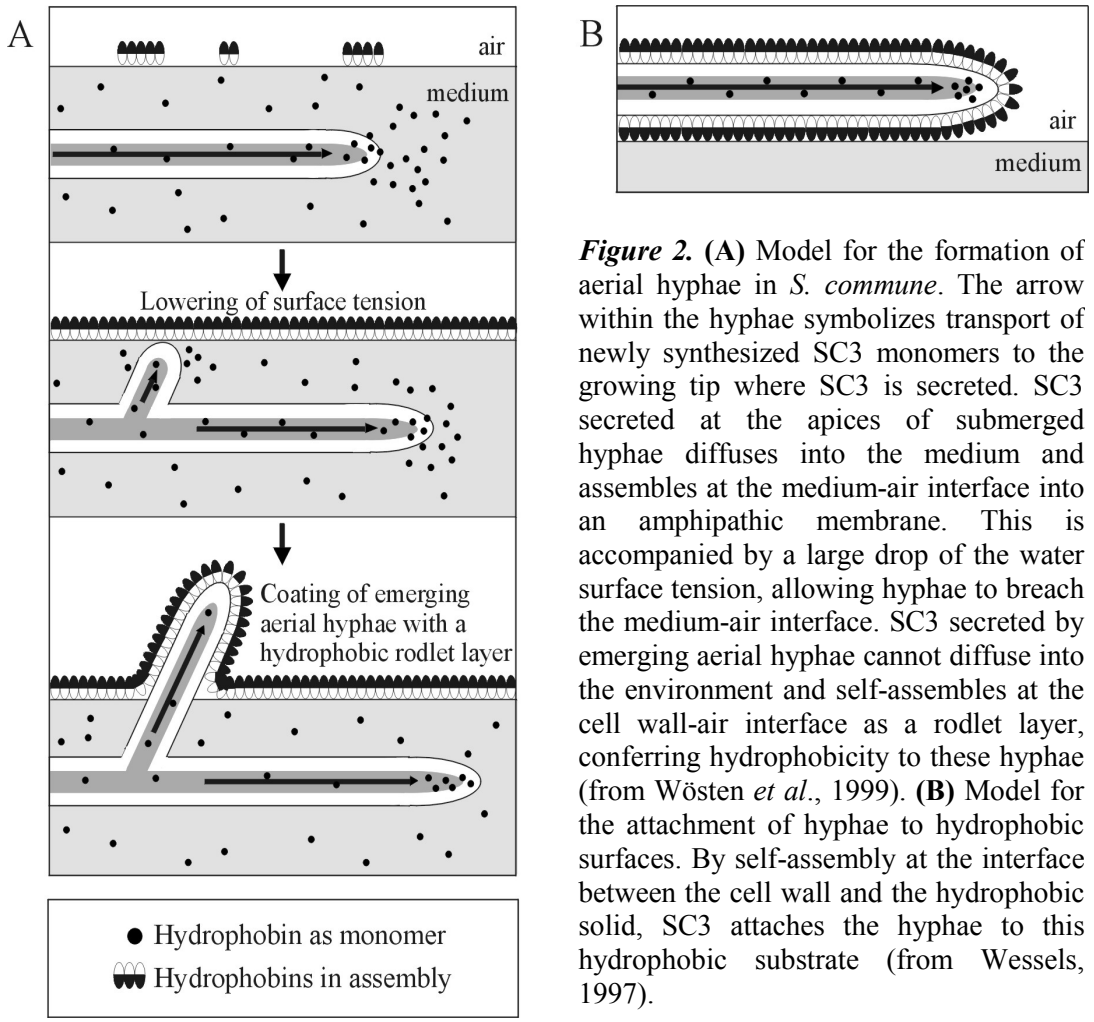

Figure 2. **(A)** Model for the formation of aerial hyphae in *S. commune*. The arrow within the hyphae symbolizes transport of newly synthesized SC3 monomers to the growing tip where SC3 is secreted. SC3 secreted at the apices of submerged hyphae diffuses into the medium and assembles at the medium-air interface into an amphipathic membrane. This is accompanied by a large drop of the water surface tension, allowing hyphae to breach the medium-air interface. SC3 secreted by emerging aerial hyphae cannot diffuse into the environment and self-assembles at the cell wall-air interface as a rodlet layer, conferring hydrophobicity to these hyphae (from Wösten *et al*., 1999). **(B)** Model for the attachment of hyphae to hydrophobic surfaces. By self-assembly at the interface between the cell wall and the hydrophobic solid, SC3 attaches the hyphae to this hydrophobic substrate (from Wessels, 1997).

SC3 secreted at the tips of aerial hyphae cannot diffuse into the medium and assembles at the interface between the hydrophilic cell wall and the hydrophobic air (Figure 2A). The hydrophilic side of the membrane faces the cell wall, while the hydrophobic side is exposed to the air, making these hyphae hydrophobic. In a strain in which the *SC3* gene was disrupted (*ΔSC3* strain), the surface tension of the medium was lowered to 45 mJ m^{-2} only and few aerial hyphae were formed. The drop in surface tension and the formation of aerial hyphae was fully restored by addition of purified SC3 hydrophobin to the culture medium. However, the aerial hyphae formed were hydrophilic and not hydrophobic like wild-type hyphae. This shows that aerial hyphae have to secrete SC3 themselves in order to be coated with a hydrophobic membrane.

In wild-type hyphae growing over a hydrophobic solid like Teflon, SC3 was localized between the cell wall and the Teflon surface. Therefore, it was proposed that SC3 is involved

in attaching the fungus to the hydrophobic surface by bridging the hydrophilic cell wall and the hydrophobic solid (Figure 2B). Indeed, it was shown that the *SC3* mutant was less firmly attached (Wösten *et al*., 1994a). In nature, attachment of *S. commune* to hydrophobic lignin may be instrumental in degradation of wood.

Recently, it was shown that SC3 not only functions at a hydrophobic-hydrophilic interface, but that it also seems to function in the matrix of the cell wall, where it may be involved in the linkage of (1-3)β-glucan to chitin (van Wetter, 2000). The *ΔSC3* strain produces large amounts of mucilage (1,3/1,6-ß-glucan) in the culture medium compared to the wild-type strain. This was correlated with a decreased amount of 1,3/1,6-ß-glucan linked to the chitin in the cell wall. Reintroducing the *SC3* gene resulted in the restoration of the wild-type cell wall composition, confirming a role of SC3 in cell wall assembly. However, the mechanism by which SC3 acts in cross-linking of wall components is not yet known.

Use of hydrophobins in medical and technical applications

The characteristic property of hydrophobins to form an amphipathic membrane upon contact with a hydrophilic-hydrophobic interface allows them to change the nature of a surface. Hydrophobic surfaces of liquids (e.g. oil droplets) or solids (e.g. Teflon) can be made hydrophilic by suspending or submerging them into a solution of hydrophobin (Wösten *et al*., 1994a; 1995; Lugones *et al*., 1996; Figure 3AB). Conversely, by allowing such a solution to evaporate on glass or filter paper, these materials become hydrophobic (Wösten *et al*., 1993; Lugones *et al*., 1996; Figure 3C). Importantly, assembled class I hydrophobins bind strongly to their supports when compared to binding of e.g. BSA (Wösten *et al*., 1993; 1994abc; 1995; Lugones *et al*., 1996; 1998). The hydrophobin layers on paper or Teflon resist washes with water or extraction with detergents at 100 °C, while BSA was completely removed from hydrophobic surfaces by treatment with hot SDS.

Because of these properties hydrophobins could be used in various medical and technical applications (Wessels, 1997). For instance, by assembling hydrophobins on their surface, the biocompatibility of medical implants could be increased or microbial cell adhesion to e.g. catheter surfaces could be prevented. Hydrophobins could also act as an priming layer to attach cells or proteins to hydrophobic surfaces, as in biosensors. Furthermore, hydrophobic solids (Teflon beads) or liquids (oils) can be stably dispersed in water by coating them with hydrophobin (Wösten *et al*., 1994a). The property of hydrophobins to coat a surface with a 10 nm thin membrane also makes these proteins interesting candidates for use in nanotechnology as defined by Thomas (1995). Because hydrophobins are ingested by humans upon consumption of mushrooms and fungus-fermented foods, hydrophobins are not expected to be toxic and can be considered as safe in food applications. In addition, preliminary data indicate that hydrophobins are not cytotoxic and not very immunogenic and can therefore be considered safe for use in medical applications (H.A.B. Wösten and M.I. Janssen, personal communication).

Genetic engineering of *SC3* can be used to study structure-function relationships of hydrophobins or to optimize their structure for use in specific applications. For instance,

solubility characteristics of the hydrophobin or the biophysical properties of the hydrophilic or hydrophobic sides of the amphipathic membrane could be changed. Assuming that the *N*-terminal part is exposed at the hydrophilic side of assembled hydrophobin, this part would be the first choice for modification. Deletion or addition of amino acid sequences would allow changes of biophysical properties of the hydrophobin or facilitate the attachment of specific molecules or cells (see Chapter 6).

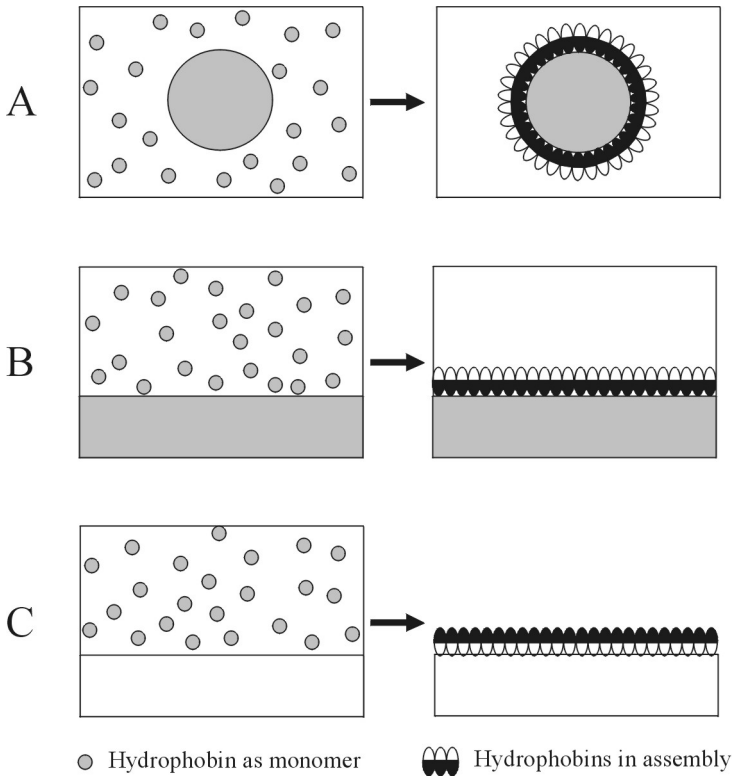

Figure 3. Hydrophobins can change the wettability of surfaces by their property to self-assemble at hydrophilic-hydrophobic interfaces. **(A)** Air bubbles or oil droplets in an aqueous solution of hydrophobin become coated with an amphipathic film that stabilizes them in water. **(B)** Similarly, when a sheet of hydrophobic plastic such as Teflon (θ 110 deg) is immersed in such a solution it is coated with a strongly adhering protein film that makes the surface wettable (θ 22-63 deg). **(C)** In contrast, hydrophobin monomers dried down on a hydrophilic surface make the surface hydrophobic (θ 110 deg)(from Wessels, 1996).

A requirement for successful application of hydrophobins is that these proteins can be produced in large quantities (i.e. grams per liter), although for some applications the quantities needed may be small (1 mg of SC3 is sufficient to coat 1 m^2 of Teflon [Wösten *et al*., 1994a]). Wild-type *S. commune* strains secrete up to 60 mg SC3 L^{-1} into the culture medium (Wösten *et al*, 1999), which makes *S. commune* the best producer of class I hydrophobins to date. Extra copies of the *SC3* gene were introduced in the wild-type strain of *S. commune* to improve production levels. However, silencing of both the introduced copies and the endogenous *SC3* gene was observed when more than one copy of the *SC3* gene were

introduced (Schuurs *et al*., 1997). Therefore, heterologous expression of hydrophobin genes was investigated to increase production levels of SC3.

Heterologous expression of proteins

Filamentous fungi can be considered as good hosts for heterologous production of proteins. Filamentous fungi, like *Aspergillus*, *Mucor*, *Penicillium*, *Rhizopus* spp. and *Trichoderma reesei*, are widely used for the commercial production of proteins because of their ability to secrete large amounts of proteins into their culture media. Species of the genera *Aspergillus* and *Trichoderma* are often used for large-scale production of proteins because of their GRAS (Generally Regarded As Safe) status and the well developed large-scale fermentation processes, down-stream processing and methods for strain improvement by classical genetic approaches, such as mutagenesis, mitotic crossing-over and screening. Furthermore, fungi glycosylate proteins in a way similar to plants and animals, which offers an advantage over the use of bacteria. Besides, production of the hydrophobins CU and POH1 was low (a few μg L^{-1}) when produced in *Escherichia coli* expression systems (Bolyard and Sticklen, 1992; Peñas *et al*., 1998). Finally, the fact that hydrophobins are fungal proteins is also an argument to explore fungal production systems.

The development of molecular biological techniques has opened new ways to use fungi for the production of homologous and heterologous proteins. It involves transformation of a host with an expression vector in which the gene encoding the desired protein is placed under control of efficient transcription regulatory sequences. This approach, in combination with classical mutagenesis and screening methods, has resulted in strains producing high amounts of homologous proteins in fermentation processes. For instance, cellulase (CBHI) in *T. reesei* (Durand *et al*., 1988) and glucoamylase in *Aspergillus* spp. (Finkelstein *et al*., 1989) were produced in quantities up to 30 g L^{-1}.

Production of heterologous proteins by filamentous fungi has been extensively reviewed (e.g. Davies, 1991; Jeenes *et al*., 1991; van den Hondel *et al*., 1991; Gwynne and Devchand, 1992; MacKenzie *et al*., 1993; Archer *et al*., 1994; Davies, 1994; Verdoes *et al*., 1995; Keränen and Penttilä, 1995; Gouka *et al*., 1997a; Archer and Peberdy, 1997). It has been proven to be most successful when proteins from related organisms are expressed. The initial level of production of heterologous fungal proteins is in the order of 10-50 mg L^{-1} (van den Hondel *et al*., 1991) although often lower levels were obtained. After optimizing the production process and applying mutagenesis and screening programs, levels in the order of grams per liter were obtained. For instance, an alkaline protease of *Fusarium* was produced to 4 g L^{-1} in *Acremonium chrysogenum* (Morita *et al*., 1994) and an aspartyl protease of *Mucor miehei* was produced to 3 g L^{-1} in *Aspergillus oryzae* (Christensen *et al*., 1988).

Levels of mammalian, bacterial, avian or plant proteins are generally low when produced in fungi (0.5-10 mg L^{-1}) (reviewed by Jeenes *et al*., 1991; van den Hondel *et al*., 1991; Gwynne and Devchand *et al*., 1992). Production can be limited at the transcriptional or the (post)-translational level, and several strategies have been developed to improve protein

yields (reviewed by Archer *et al*., 1994; Archer *et al*., 1997; Gouka *et al*., 1997b). Most of these strategies are similar to those used for fungal proteins (Verdoes *et al*., 1995) and include: (i) the introduction of a large number of gene copies, (ii) the use of strong fungal transcription regulatory sequences and efficient secretion signals, (iii) the construction and use of protease deficient host strains, (iv) development of an optimal production medium and (v) gene fusion strategies in which the gene of interest is fused at the 3' end of a gene encoding a well-secreted homologous protein. Additional strategies to improve heterologous protein production include optimization of codon usage, removal of AU-rich sequences to prevent incorrect mRNA processing and overexpression of chaperones and/or foldases to aid folding of the protein of interest. Some of these strategies will be discussed in the next sections.

Optimization of heterologous protein production at the transcriptional level

The most straightforward approach to enhance the transcription level of the introduced gene is to increase its copy number. Analysis of *Aspergillus niger* multicopy transformants for glucoamylase showed a gene dosage dependent expression of glucoamylase up to about 20 copies (Verdoes *et al*., 1993). Studies with the glucoamylase promoter fused to a reporter gene suggested that transcription in multi-copy transformants was limited due to titration of trans-acting regulatory proteins (Verdoes *et al*., 1994a) and was also suggested to be a limiting factor in expression of other fungal genes (Gwynne *et al*., 1987; Kelly and Hynes, 1987; Andrianopoulos and Hynes, 1988; Beri *et al*., 1990; Burger *et al*., 1991; Margolles-Clark *et al*., 1996). In some cases this was overcome by increasing the expression level of the regulatory gene as well (Kelly and Hynes, 1987; Beri *et al*., 1990; Burger *et al*., 1991). Analysis of production levels of transformants containing a similar number of gene copies suggests that the site of integration also affects the expression of the introduced gene (Ward *et al*., 1990; Verdoes *et al*., 1993; 1994b). The effect of random integration can be overcome by targetting the expression vector to loci of known high transcriptional activity, like to the loci for glucoamylase and CBHI in *A. niger* and *T. reesei*, respectively. Harkki *et al*. (1991) and Nyyssönen and Keränen (1995) reported that in transformants giving the best production of endoglucanase I (EGI) and antibody Fab fragments, respectively, the expression constructs were integrated into the *CBHI* locus.

Limitation of heterologous protein production at the transcriptional level can also be caused by incorrect processing of pre-mRNA and/or a low mRNA stability. When wild-type α-galactosidase (*aglA*) was expressed in *A. niger* a truncated transcript was observed that contained only 200 bp of the *aglA* gene (Gouka *et al*., 1996). The truncated transcript was observed in *A. niger* and *Aspergillus nidulans* but not in *Bacillus subtilis* (Overbeeke *et al*., 1990), *Hansenula polymorpha* (Fellinger *et al*., 1991), *Saccharomyces cerevisiae* (Verbakel, 1991) and *Kluyveromyces lactis* (Bergkamp *et al*., 1992). By replacing an AT-rich sequence in the *aglA* gene with a more GC-rich sequence ($aglA_{syn}$), a full-length *aglA* transcript was produced in *A. niger*. Incorrect processing of heterologous genes was also shown to occur in *Pichia pastoris* (Scorer *et al*., 1993), *Cryptococcus curvatus* (J. Springer, unpublished) and *S.*

commune (Schuren *et al*., 1998). For instance, truncated transcripts of various reporter and selection genes (e.g. the *hygromycin B resistance* gene) were observed in *S. commune* (Schuren *et al*., 1998). The results suggested that AT-rich sequences in the coding region of heterologous genes acted as internal polyadenylation sequences. The mechanisms for recognizing polyadenylation signals (or AU-rich stretches in transcripts) are unclear and may vary between organisms and genes to be expressed. Therefore, the occurrence of premature termination cannot be predicted but should be considered when expression of the target gene is absent.

Low mRNA stability can be partially overcome by fusing the gene of interest to the 3' end of a homologous gene. For instance, by fusing human interleukin-6 (*hil6*) or α - galactosidase ($aglA_{syn}$) genes behind the glucoamylase gene, mRNA levels of these genes increased compared to that of non-fused genes (Gouka *et al*., 1996).

Optimization of heterologous protein production at the (post)-translational level

The secretion pathway of proteins is complex and involves several cellular compartments. Ultrastructural electronmicroscopic studies of filamentous fungi suggest that the processes in these organisms do not essentially differ from those in yeast and mammalian cells that have been extensively studied (Rothblatt *et al*., 1994; Punt *et al*., 1994; Archer and Peberdy, 1997; Gouka *et al*., 1997a). Secretion of proteins is considered to involve the processes of (i) translation and translocation across the endoplasmic reticulum (ER) membrane into the ER-lumen and the removal of the signal peptide from the newly synthesized protein. (ii) Quality control in the ER, which involves the combined action of chaperones (e.g. binding protein and calreticulin/calnexin/UDP-Glc transferase complex) and foldases (e.g. protein disulphide isomerase and peptidyl prolyl *cis-trans* isomerases). The chaperones and foldases assist in the folding of newly synthesized proteins and they prevent transport of proteins to other compartments before they are folded properly. Incorrectly folded proteins are recognized by an unknown mechanism and are degraded by proteases in the cytoplasm. As in other eukaryotes this process probably involves ubiquitin labeling of the protein (Coux *et al*., 1996) and transport from the ER lumen to the cytoplasm (Wiertz *et al*., 1996). (iii) Transport from the ER to the Golgi equivalent (a recognizable Golgi apparatus is absent in fungi) where glycosylation and processing (e.g. cleavage of propeptides from the mature protein) occur and (iv) transport from the Golgi to the plasmamembrane via vesicles. These vesicles fuse with the membrane and extrude their contents into the cell wall. The proteins may then be carried to the outside of the wall by the flow of wall constituents at the growing hyphal apex (Wessels, 1993) and released into the medium.

Fusion of the gene to be expressed behind a highly expressed homologous gene does not only increase stability of the mRNA transcript (see above) but can also resolve limitations at early stages in the secretion pathway (ER). Using glucoamylase of *A. niger* or cellobiohydrolase (CBHI) of *T. reesei* as a carrier increased levels of secreted protein 5-1000 fold to 5-250 mg L^{-1} (Ward *et al*., 1990; Contreras *et al*., 1991; Roberts *et al*., 1992; Broekhuijsen *et al*., 1993; Jeenes *et al*., 1993; Nyyssönen and Keränen, 1995; Ward *et al*.,

1995). The carrier improves translocation of the protein into the ER and allows proper folding, thereby protecting the heterologous protein from degradation. In most cases the fusion protein is cleaved at a later stage in the secretory pathway. Cleavage can occur by autocatalytic processing of the heterologous protein (Ward *et al*., 1990), by an unknown fungal protease (Roberts *et al*., 1992; Baron *et al*., 1992; Nyyssönen *et al*., 1993; Nyyssönen and Keränen, 1995) or by a KEX2-like protease for which a recognition site is introduced in the fusion protein (Contreras *et al*., 1991; Broekhuijsen *et al*., 1993; Ward *et al*., 1995). Another strategy to improve transport of proteins through the secretion pathway is overexpression of foldases and chaperones (Gouka *et al*., 1997a and references therein).

Low production levels of heterologous proteins can also be caused by the action of proteases. Extracellular proteases of *Aspergillus*, such as aspergillopepsin (Berka *et al*., 1990), are responsible for the degradation of many heterologous proteins (Archer *et al*., 1992; Broekhuijsen *et al*., 1993). Fungal strains deficient in extracellular proteases have been isolated by random mutagenesis (Mattern *et al*., 1992; van den Hombergh *et al*., 1995, 1997) or molecular genetic approaches (Berka, 1990; van den Hombergh *et al*., 1997). The use of these protease deficient strains has resulted in improvement of production levels of heterologous proteins (Berka, 1991; Roberts *et al*., 1992; Broekhuijsen *et al*., 1993). Intracellular or cell-wall localized proteases can also be responsible for low yields of secreted heterologous proteins as in the case of hIL-6 in *Aspergillus awamori* (Gouka *et al*., 1996). In *S. cerevisiae*, the use of strains deficient in vacuolar proteases has led to increased levels of heterologous proteins (Suzuki *et al*., 1989; Wingfield and Dickinson, 1993).

Another way to improve production of heterologous proteins is medium development (Smith and Wood, 1991) and controlled, large-scale fermentation (reviewed by Greasham, 1991; Dunn-Coleman, 1992). For a number of fungal proteins (e.g. glucoamylase) the fermentation conditions have been optimized. The use of heterologous expression signals, like those of the glucoamylase gene, might allow high level production of proteins under already optimized culture conditions, as was shown for phytase (van Gorcom *et al*., 1990) and aspartic protease (Ward and Kodama, 1991). It has been suggested (Verdoes *et al*., 1995) that the use of a promoter, which is induced under conditions that genes for proteolytic enzymes are repressed, may also be advantageous.

Outline of this thesis

The primary aim of the research described in this thesis was to improve the production level of the SC3 hydrophobin of *S. commune* by heterologous expression in *A. niger* and *T. reesei* and to use genetic engineering of SC3 to study structure-function relationships in hydrophobins and to optimize them for use in specific applications. In the course of this work the phenomenon of intron-dependent mRNA accumulation was discovered when *S. commune* was transformed with genomic or cDNA sequences. In addition, it was shown that the GC-content of genes also determined expression.

Chapter 2 deals with the heterologous expression of *SC3* in *A. niger* and *T. reesei*. *A. niger* was initially chosen because this fungus does not secrete hydrophobins into the culture medium, simplifying purification procedures. *SC3* was expressed in wild-type and protease deficient *A. niger* strains behind the *gpd* promoter of *A. nidulans* or as a fusion with the glucoamylase gene (G2) of *A. niger*. In all cases the level of SC3 produced was low (less than 1% of the amount produced by *S. commune*), suggesting that *A. niger* is not a suitable host for expression of hydrophobin genes. *SC3* was then expressed in *T. reesei* using the *SC3* coding sequence and the *HFBI* or the *HFBII* promoter. *HFBI* and *HFBII* encode class II hydrophobins that are abundantly secreted into the culture medium of *T. reesei*. In both cases the amount of SC3 obtained was at least similar to that in *S. commune*.

Chapter 3 describes a sandwiched-culture technique, which can be used to screen filamentous fungi for production of heterologous proteins. This technique involves culturing transformants between perforated polycarbonate membranes and transferring this sandwiched-culture to a PVDF membrane. Secreted proteins are then immobilized on the PVDF membrane and can be monitored by immunodetection. This technique allows detection of proteins that otherwise may be degraded by proteases in the medium.

Chapter 4 describes the phenomenon of intron-dependent mRNA accumulation in *S. commune*. It was shown that mRNA did not accumulate when homologous and heterologous cDNA sequences were introduced in *S. commune*. Addition of artificial or naturally occurring introns restored mRNA accumulation. Run-on analysis with nuclei harboring intron-containing and intron-less sequences showed that this effect did not occur at the level of transcription initiation: genomic and cDNA sequences were equally active in this respect.

In **Chapter 5** it is shown that expression of the bacterial *hygromycin B resistance* gene in *S. commune* depends on the GC-content of the gene. It was suggested before that the presence of an AT-rich region in the 5' part of the gene causes truncation of the RNA transcript (Schuren and Wessels, 1998). Increasing the GC-content in this region restored formation of full-length transcripts and resulted in hygromycin B resistance when introduced in *S. commune*. Accumulation of mRNA of the *hygromycin B resistance* gene was also depended on the presence, number and/or the position of introduced introns.

In **Chapter 6** it is shown that the hydrophilic side of assembled SC3 can be modified by changing the *N*-terminal part of the protein. Self-assembly of SC3 was not affected by deleting 25 *N*-terminal amino acids of the mature protein or by introducing the cell-binding domain of human fibronectin (RGD) at the *N*-terminus. However, the modifications did change the physiochemical properties of the hydrophilic side of assembled SC3. Removal of the 25 amino acids at the *N*-terminus of SC3 decreased the wettability of the hydrophilic side of the amphipathic membrane. The decrease in wettability of the hydrophilic side of the amphipathic membrane and the introduction of the RGD sequence at the *N*-terminus both were shown to stimulate growth of fibroblasts at Teflon surfaces coated with the SC3 derivatives when compared to bare Teflon and Teflon coated with unmodified SC3.

In **Chapter 7** the results presented in this thesis are summarized and discussed.